Amino terminal region of acute phase, but not constitutive, serum amyloid A (apoSAA) specifically binds and transports cholesterol into aortic smooth muscle and HepG2 cells

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Abstract The human apoSAA proteins comprise both acute phase ($apoSAA_1$, $apoSAA_2$) and constitutive ($apoSAA_4$) isoforms; all are expressed in human atherosclerotic lesions as well as in liver. Recombinant acute phase apoSAA binds cholesterol with an affinity of ~170 nM and enhances cholesterol uptake by HepG2 cells (*J. Lipid Res.* 1995. **36:** 37–46). In the present study, we sought to define the region of acute phase apoSAA involved in cholesterol binding and to investigate the ability of constitutive $apoSAA_4$ to bind cholesterol. Binding of [^{3}H]cholesterol to $apoSAA_p$ was inhibited by unlabeled cholesterol (1–100 nM), but not significantly by vitamin D and estradiol. Direct binding of acute phase, but not constitutive, apoSAA to the surfaces of polystyrene microtiter wells was strongly diminished in the presence of cholesterol. The ability of $apoSAA_p$ to bind cholesterol was inhibited by antibodies to human $apoSAA_1$ and to peptide 1–18 of $apoSAA_1$. There was only slight inhibition of cholesterol binding by antibodies to peptide 40–63, and no inhibition by antibodies to peptides spanning regions containing amino acid residues 14–44 and 59–104. [^{3}H]cholesterol uptake by neonatal rabbit aortic smooth muscle and HepG2 cells was enhanced by a synthetic peptide corresponding to amino acids 1–18 of $hSAA_1$, but not by peptides corresponding to amino acids 1–18 of $hSAA_4$. [^{3}H]cholesterol uptake by HepG2 cells was slightly increased by a peptide corresponding to amino acids 40–63 of $hSAA_1$. These findings suggest that apoSAA modulates the local flux of cholesterol between cells and lipoproteins during inflammation and atherosclerosis.—**Liang, J-s., B. Schreiber, M. Salmona, G. Phillip, W. A. Gonnerman, F. C. de Beer, and J. D. Sipe.** Amino terminal region of acute phase, but not constitutive, serum amyloid A (apoSAA) specifically binds and transports cholesterol into aortic smooth muscle and HepG2 cells. *J. Lipid Res.* 1996. **37:** 2109–2116.

Supplementary key words serum amyloid A isoforms • lipoproteins • vitamin D • estradiol

Serum amyloid A (apoSAA) proteins are a family of apolipoproteins comprising a number of closely related, cytokine-inducible acute phase isoforms and also a single constitutive isoform (1, 2). Although the structure and catabolism of the apoSAA proteins have been investigated in terms of their precursor relationship to the amyloid A fibrils associated with secondary amyloidosis, the normal physiological function of apoSAA remains unclear. The constitutive isoform $apoSAA_4$, a relatively minor apolipoprotein of high density lipoprotein (HDL), comprises more than 90% of total apoSAA present on HDL in the absence of inflammation. However, within a few hours of tissue injury and cell necrosis, the amount of acute phase isoforms on HDL is increased by several hundred fold while the amount of apolipoprotein A-I (apoA-I) is decreased, and, for a time, the amount of acute phase apoSAA can be comparable to or more than apoA-I (1). Both of the acute phase isoforms, $apoSAA_1$ and $apoSAA_2$, as well as $apoSAA_4$ are expressed in human atherosclerotic lesions (3).

Epidemiologic data indicate an inverse relationship between HDL concentration and the risk of cardiovascular disease. Thus, an alteration of homeostatic HDL composition may result in disturbance of cholesterol homeostasis and an increased risk of atherosclerosis (4, 5). For example, as apoA-I serves as a cofactor for LCAT (6) and is important for cholesterol efflux from cells, the replacement of apoA-I by apoSAA on HDL might be expected to compromise reverse cholesterol transport.

Recently, we reported that apoSAAp, a recombinant generated hybrid of $apoSAA_1$ and $apoSAA_2$, binds cho-

Abbreviations: AA, amyloid A; apoSAA, serum amyloid A; apoA-I, apolipoprotein A-I; DMEM, Dulbecco's modified Eagle's medium; HDL, high density lipoprotein; hSAA, human serum amyloid A; RPMI, Roswell Park Memorial Institute; SMC, smooth muscle cells.

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lesterol with an affinity of ~170 nM, and apparently forms a dimer upon cholesterol binding. Furthermore, the addition of apoSAAp to cultures of HepG2 cells in lipoprotein-deficient medium resulted in an enhanced cellular uptake of cholesterol (7). These initial findings suggested that acute phase apoSAA may interact directly with cholesterol and thus alter the normal mechanism of homeostatic reverse cholesterol transport by HDL. The present study was undertaken to define the domain of acute phase apoSAA responsible for cholesterol binding and transport, and to determine the ligand specificity of cholesterol binding. In order to determine whether production of apoSAA might play a role in the genesis of atherosclerotic lesions, the effect of recombinant acute phase $apoSAA_p$ and synthetic peptide fragments of acute phase and constitutive apoSAA on cholesterol uptake by neonatal rabbit aortic smooth muscle cells (SMC) was also measured.

MATERIALS AND METHODS

Materials

Recombinant synthetic human $apoSAA_p$ was purchased from Pepro Tech (Rocky Hill, NJ). $ApoSAA_p$ is a hybrid molecule corresponding to human $apoSAA_{1\alpha}$ except for the N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71; the latter two substituted residues are present in $apoSAA_{2\beta}$. Lyophilized $apoSAA_p$ was dissolved in water at concentrations of 100 μg/ml and stored at -20°C. Immediately before use, the stock solution was diluted with serum-free DMEM. Human $apoSAA_4$ was purified from plasma as described (8). [4-^{14}C]cholesterol (57.1 mCi/mmol) and [1,2,6,7-^{3}H(N)]cholesterol (101 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). Cholesterol, vitamin D, and estradiol, purchased from Sigma Chemical Co. (St. Louis, MO), were dissolved in ethanol at a concentration of 10 mg/ml, respectively, and stored at -20°C. All other chemicals were reagent grade.

Synthetic peptides corresponding to residues 1–18 ($apoSAA_1$ 1–18) and 40–63 ($apoSAA_1$ 40–63) of human $apoSAA_1$ ($hSAA_1$), and residues 1–18 ($apoSAA_4$ 1–18) of human $apoSAA_4$ ($hSAA_4$) were synthesized by solid phase chemistry with a model 430A synthesizer from Applied Biosystems (Foster City, CA) and purified by reverse-phase HPLC (model 243, Beckman Instruments Inc., Palo Alto, CA) on a Delta-Pak C18 column (19 × 300 mm, 300A pore size, 15 μm particle size, Nihon Waters, Tokyo, Japan) using a linear gradient of 100% water + 0.1% trifluoroacetic acid to 100% acetonitrile + 0.1% trifluoroacetic acid over 90 min. Their purity and identity were determined by analytical reverse phase high pressure liquid chromatography, capillary electrophoresis (Quanto 4000, Millipore, Bedford, MA), and amino acid sequencing (Applied Biosystems 477A microsequencer); purity of peptides was hSAA1 1–18, 96%; hSAA1 40–63, 92%, and hSAA4 1–18, 90%. Lyophilized peptides were dissolved in DMSO at concentrations of 100 μg/ml and stored at -20°C. Immediately before use, the stock solution was diluted with serum-free Dulbecco's modified Eagle's medium (DMEM) to desired concentrations. Rabbit polyvalent antibodies raised to human acute phase $apoSAA_1$ and to synthetic peptides whose amino acid sequence correspond to eight overlapping regions, 1–18, 14–30, 27–44, 40–63, 59–72, 68–84 and 89–104 of $apoSAA_1$ as previously described (8) were used. It was established by Western blotting experiments that each of the anti-peptide antibodies binds to $apoSAA_p$.

Radioiodination of apoSAAp and $apoSAA_4$

ApoSAAp or $apoSAA_4$, at a concentration of 1 mg/ml in 0.15 M NaCl, pH 7.4, was combined with ICl reagent in 0.1 M glycine as previously described (7). After reaction for 10 min with 1 mCi Na ^{125}I at room temperature, the free iodide was removed by gel filtration using Sephadex G-25. The specific radioactivities of ^{125}I-labeled apoSAAp and ^{125}I-labeled $apoSAA_4$ were 0.26 mCi/mg and 0.35 mCi/mg, respectively.

ApoSAA direct binding assay

^{125}I-labeled apoSAAp or ^{125}I-labeled $apoSAA_4$ was diluted with serum-free Roswell Park Memorial Institute (RPMI) medium to a concentration of 10 pmol/ml. Triplicate aliquots (100 μl) were incubated in microtiter wells at 37°C for up to 6 h. The amount of ^{125}I-labeled $apoSAA_p$ or ^{125}I-labeled $apoSAA_4$ that was directly bound to polystyrene microtiter wells at each time point was determined by counting triplicate 10-μl aliquots of solution. The bound radioactivity was determined as the difference between the amount of radioactivity that was added and what remained unbound after varying lengths of incubation.

Cholesterol binding assay

ApoSAAp (20 pmol) was incubated at 25°C for 18 h in 1 ml serum-free RPMI (Roswell Park Memorial Institute) medium containing 20 pmol [^{3}H]cholesterol in the presence or absence of 1–100 pmol unlabeled cholesterol, vitamin D, or estradiol. Cholesterol-apoSAA complexes were isolated by rapid filtration through nitrocellulose membranes (0.45 μm, Millipore Corp, Bedford, MA) followed by washing ten times with 10 ml of ice-cold PBS, and drying at room temperature. ApoSAAp is retained by nitrocellulose membranes, whereas free cholesterol, vitamin D, and estradiol are washed through. The quantity of apoSAA bound cholesterol

retained on the membrane was determined by liquid scintillation counting (7).

Antibody blocking of cholesterol binding

In order to test the ability of antibodies to the entire molecule or to seven overlapping regions corresponding to amino acids 1–18, 14–30, 27–44, 40–63, 59–72, 68–84 and 89–104 of apoSAA to block the binding of cholesterol by apoSAA, apoSAAp (167 pmol) in 1 ml serum-free RPMI medium was preincubated with or without antibodies at dilutions of 1:500 to 1:5000 at 37°C for 30 min. The apoSAA–antibody mixtures were then incubated with [^{3}H]- or [^{14}C]cholesterol (250 pmol) at 25°C for 18 h and apoSAA-bound cholesterol was isolated and quantitated as described above.

Neonatal rabbit aortic SMC and HepG2 cells

Neonatal rabbit aortic SMC was isolated from aortae of 3-day-old New Zealand white rabbits (Pine Acres Rabbitry, Brattleboro, VT) as described previously (9). Essentially, the intima was removed from aortae which were minced and digested with collagenase and porcine pancreatic elastase. Cells (5×10^5) were seeded in a 25 cm^2 tissue culture flask in DMEM (J.R.H. Biosciences, Lenexa, KS) containing 3.7 g/L $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (DMEM), and 20% fetal bovine serum (FBS, Sigma Chemical Co.). The primary stage cells were maintained for 1 week and were then passaged after trypsinization with 0.5% trypsin/0.02% EDTA and maintained in DMEM containing 10% FBS for 1 week at which time cells were passaged into 24-well microtiter plates at a density of 40,000 per well and maintained in DMEM containing 10% FBS for 2–3 weeks. HepG2 cells were seeded at a density of 1×10^5 cells/well and propagated in monolayer culture at 37°C, 5% CO_2 for 2–4 weeks in 24-well microtiter plates using DMEM plus 10% FBS and 50 μg/ml gentamycin.

Cholesterol uptake by SMC and HepG2 cells

The second passage SMC were washed with serum-free DMEM medium, and 0.4 ml medium containing [^{3}H]cholesterol in the presence or absence of synthetic peptides, apoSAAp, or BSA was added to each of triplicate cell culture wells and incubated for 24 h. After incubation, the culture medium was removed and each well was washed three times with serum-free DMEM medium. Each well received 0.5 ml Gel and Tissue Solubilizer and was incubated at 70°C for 4 h. Triplicate 50-μl aliquots of the homogenates were taken to measure radioactivity and triplicate 10-μl aliquots were taken for protein assay. Protein was measured colorimetrically using a kit purchased from Pierce Chemical (Rockford, IL).

Secondary structure analysis

The secondary structure analysis of apoSAA$_1$ and apoSAA$_4$ according to the method of Garnier, Osguthorpe, and Robson (10) was carried out by using the PCGene Software, IntelliGenetics, Inc., CA.

Statistical analysis

Experimental groups were compared by one-factor and two-factor analyses of variance using the ANOVA statistical package in SAS software.

RESULTS

Comparison of the interaction of ^{125}I-labeled apoSAA$_p$ and ^{125}I-labeled apoSAA$_4$ with cholesterol as determined by the direct binding assay

^{125}I-labeled apoSAA$_p$ or ^{125}I-labeled apoSAA$_4$ was diluted with serum-free RPMI medium to a concentration of 10 pmol/ml. Triplicate aliquots (100 μl) were incubated in microtiter wells at 37°C for up to 6 h. The amount of ^{125}I-labeled apoSAA$_p$ or ^{125}I-labeled apoSAA$_4$ that was directly bound to polystyrene microtiter wells at each time point was determined as the difference between the amount of radioactivity that was added and the amount remaining in solution determined by counting 10-μl aliquots from triplicate wells. Within 6 h of incubation at 37°C, 0.80 ± 0.09 pmol of ^{125}I-labeled apoSAA$_p$, but only 0.40 ± 0.04 pmol of ^{125}I-labeled apoSAA$_4$, was bound to the surface of polystyrene microtiter wells (**Fig. 1**). Binding of ^{125}I-labeled apoSAA$_p$, but not of ^{125}I-labeled apoSAA$_4$, was significantly diminished (apoSAA$_p$: from 0.8 to 0.52 pmol, $P < 0.05$; apoSAA$_4$: from 0.40 to 0.31 pmol, $P > 0.05$) in the presence of cholesterol (20 μg/ml) (Fig. 1).

Secondary structure analysis

The first 11 of the amino terminal amino acids of apoSAA were found by secondary structure predictive analysis to be important in lipid binding and to formation of amyloid fibrils in vitro (11, 12). A comparison of the primary structures of apoSAA$_1$ and apoSAA$_4$ in the amino-terminal region of apoSAA$_1$ spanning residues 1–11 is shown in **Fig. 2.** The differences between apoSAA$_1$ and apoSAA$_4$ in this region occur at residue 1, R in apoSAA$_1$, E in apoSAA$_4$; residues 3 and 4, –FF– in apoSAA$_1$, –WR– in apoSAA$_4$; residues 7 and 8, –LG– in apoSAA$_1$, –FK– in apoSAA$_4$, and at residue 11, F in apoSAA$_p$, L in apoSAA$_4$ in the amino-terminal 1–11 region. Analysis of the secondary structure of the polypeptide segment comprised of the region spanning amino acids 1–11 by the method of Garnier, Osguthorpe, and Robson (10) revealed that there is a more extended (55%), but less helical (36%) conformation in

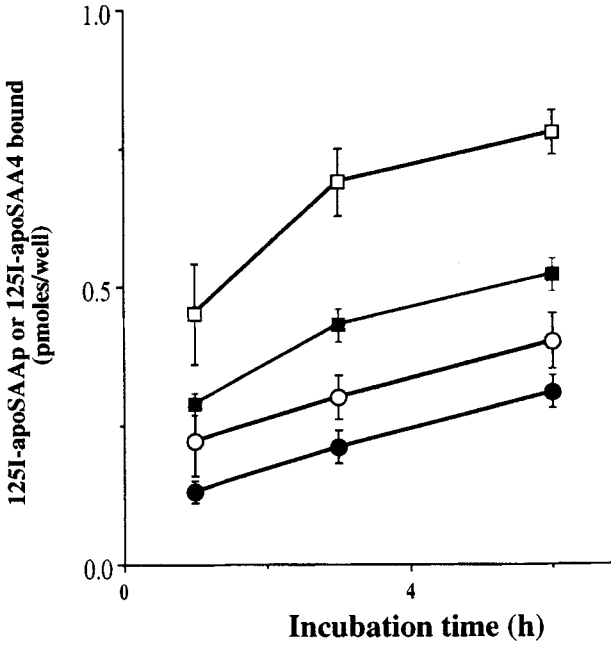

Fig. 1. Influence of cholesterol on the time course of direct binding ^{125}I-labeled apoSAA$_p$ and ^{125}I-labeled apoSAA$_4$ to microtiter wells. One hundred µl of ^{125}I-labeled apoSAA$_p$ (10 pmol/ml) or ^{125}I-labeled apoSAA$_4$ (10 pmol/ml) in serum-free RPMI medium with cholesterol (20 µg/ml) (apoSAAp + cholesterol: ■; apoSAA$_4$ + cholesterol: ●) or without cholesterol (apoSAA$_p$ alone: □; apoSAA4 alone: ○) were added to each well and incubated at 37°C. At intervals of 1, 3, and 6 h, 20 µl of culture media was removed for counting of radioactivity. The bound radioactivity was determined as the difference between the amount of radioactivity added and that remaining after incubation. The data are expressed as mean ± standard deviation (SD). Experiment was performed four times.

apoSAA$_1$ as compared to the 100% helical conformation in apoSAA$_4$ (Fig. 2).

Lack of interaction of vitamin D and estradiol with acute phase apoSAA

ApoSAA$_p$ (20 pmol) was incubated at 25°C for 18 h with 1 ml serum-free RPMI medium containing 20 pmol [^{3}H]cholesterol in the presence or absence of unlabeled cholesterol, vitamin D, or estradiol. Cholesterol-apoSAA complexes were isolated by rapid filtration through nitrocellulose membranes and the quantity of radioactive cholesterol was determined by liquid scintillation counting. Binding of [^{3}H]cholesterol to apoSAA$_p$ was inhibited in a dose-dependent manner by unlabeled cholesterol (1–100 nM). Inhibition of binding by either vitamin D, which contains the aliphatic side chain of cholesterol, or estradiol, which contains the steroid nucleus but lacks this side chain, was neither dose-dependent nor statistically significant (**Fig. 3**). The lack of competition of cholesterol binding by vitamin D and estradiol suggests that cholesterol binding by apoSAA is specific with respect to ligand structure.

Localization of the cholesterol binding region within apoSAA$_p$

In order to evaluate the capacity of antibodies to specific regions of apoSAA to block cholesterol binding, apoSAA$_p$ (167 pmol) was preincubated in presence of antibodies to the entire apoSAA molecule (1–104) or seven overlapping regions corresponding to amino acids 1–18, 14–30, 27–44, 40–63, 59–72, 68–84 and 89–104 of apoSAA at 37°C for 30 min in 1 ml serum-free RPMI medium. The apoSAA–antibody mixtures were then incubated with radiolabeled cholesterol (250 pmol) at 25°C for 18 h. Bound cholesterol was measured as described in Materials and Methods. The data in **Fig. 4A** show that only antibodies to the entire apoSAA molecule (1–104) or to the 1–18 or 40–63 regions of apoSAA inhibited cholesterol binding by apoSAA. Antibodies to the other five overlapping regions corresponding to amino acids 14–30, 27–44, 59–72, 68–84 and 89–104 of apoSAA, however, did not inhibit cholesterol binding by apoSAA, although all of these antibodies reacted with apoSAA in Western blotting experiments. In further studies (Fig. 4B), the data confirm the ability of antisera to block cholesterol binding by apoSAA in experiments in which increasing concentrations of antisera were more effective in blocking cholesterol binding to the entire apoSAA molecule (1–104) or to the 1–18 or 40–63 regions of apoSAA. In control experiments, the binding of cholesterol by apoSAA$_p$ was not inhibited by antibodies to rabbit anti-mouse amyloid A, and there was no binding of radiolabeled cholesterol by the antibodies at dilutions of 1:500, 1:1000, and 1:5000 in the absence of apoSAA$_p$ (data not shown).

Enhancement of cholesterol uptake by SMC and HepG2 cells

To investigate the potential pathophysiologic significance of cholesterol binding by apoSAA, the effects of apoSAA$_p$ and synthetic peptides corresponding to the cholesterol binding region of acute phase apoSAA and the corresponding region of constitutive apoSAA on cholesterol uptake by SMC and HepG2 cells was investigated. SMC and HepG2 cells were washed thrice with serum-free DMEM, and then 0.4 ml media containing [^{3}H]cholesterol alone or with increasing concentrations of synthetic peptides, apoSAA$_p$, or BSA were added to each of triplicate wells and incubated for 24 h at 37°C. The quantity of cholesterol uptake by the cells was determined as described in Materials and Methods. The data in **Fig. 5A** show that the addition of apoSAA$_p$ (2 µM) to the medium enhanced cholesterol uptake by HepG2 cells 2- to 3-fold over basal levels as previously shown (7). Likewise, synthetic peptides corresponding

Isoforms		Residues
		1 10 11
apoSAA1	Primary structure	RSFFSFLGEA F
	Secondary structure	EEEEEECHHH H
		1 10 11
apoSAA4	Primary structure	ESWRSFFKEA L
	Secondary structure	HHHHHHHHHH H

Fig. 2. A comparison of the primary and predicted secondary structures of apoSAA$_1$ and apoSAA$_4$ in the amino-terminal region spanning residues 1–11. Secondary structure was predicted by the method of Garnier et al. (10). C: coil conformation; E: extended conformation; H: helical conformation.

to hSAA$_1$ 1–18 and 40–63 were capable of increasing cholesterol uptake by HepG2 cells (Fig. 5A).

In contrast to the stimulatory effect of hSAA1 1–18 on cholesterol uptake by HepG2 cells, the amino terminal peptide of constitutive SAA, hSAA$_4$ 1–18, at concentrations of 0.2–20 μM did not alter cholesterol uptake (Fig. 5A). In our further studies, the data in Fig. 5B show that cholesterol uptake by SMC was also enhanced by either apoAAp or a synthetic peptide corresponding to hSAA$_1$ 1–18 at 2 μM, but not significantly by BSA or a synthetic peptide corresponding to hSAA$_1$ 40–63.

DISCUSSION

Our results have demonstrated, at physiological pH (7.2) and ionic strength, that acute phase apoSAA, but not constitutive, apoSAA$_4$ binds cholesterol at the amino terminal region. The potential biological significance of this binding was established using synthetic peptides corresponding to residues 1–18 of human SAA$_1$. Like apoSAA$_p$, a synthetic peptide corresponding to residues 1–18 of human SAA$_1$ enhances cholesterol uptake by rabbit aortic SMC as well as by HepG2 cells, whereas the corresponding peptide from apoSAA$_4$ has no effect. These findings suggest that the amino terminal region of acute phase apoSAA binds and transports cholesterol into SMC and HepG2 cells. Thus, apoSAA may modulate the flux of cholesterol between cells and lipoproteins during the acute phase response and atherosclerosis. The accumulation of intracellular cholesterol is a hallmark of atherosclerosis and apoSAA may act to contribute to SMC foam cell formation. Mitchell and Brinckerhoff's study (13) suggests that rabbit fibroblasts possess high affinity binding sites for rabbit apoSAA$_3$. Our preliminary, unpublished observations

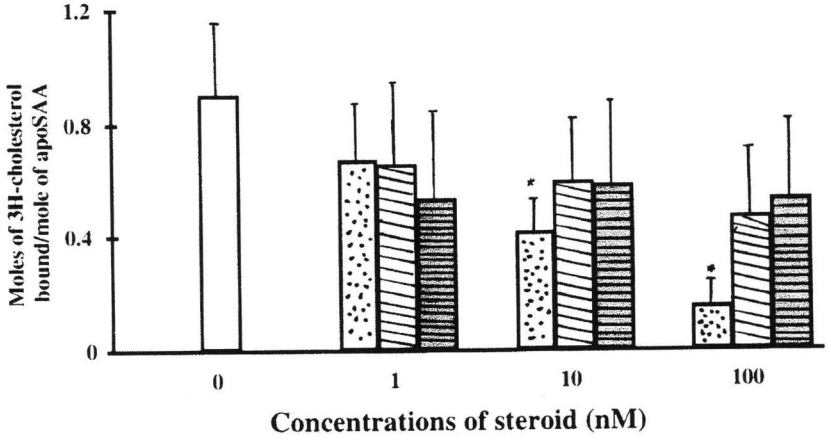

Fig. 3. Inhibition of [^{3}H]cholesterol binding to apoSAA by unlabeled cholesterol, but not by vitamin D and estradiol. ApoSAA$_p$ (20 pmol) was incubated at 25°C for 18 h with 1 ml serum-free RPMI medium containing 20 pmol [^{3}H]cholesterol in the presence of 1–100 pmol of unlabeled cholesterol (□), vitamin D (□) or estradiol (□). Cholesterol–apoSAA complexes were isolated and measured as described in Materials and Methods. Comparison of the data by two-factor analysis of variance demonstrates that both steroid and dose significantly alter cholesterol binding by apoSAA. The data are expressed as mean ± standard deviation (SD). This experiment was performed four times. *$P < 0.05$ compared to control.

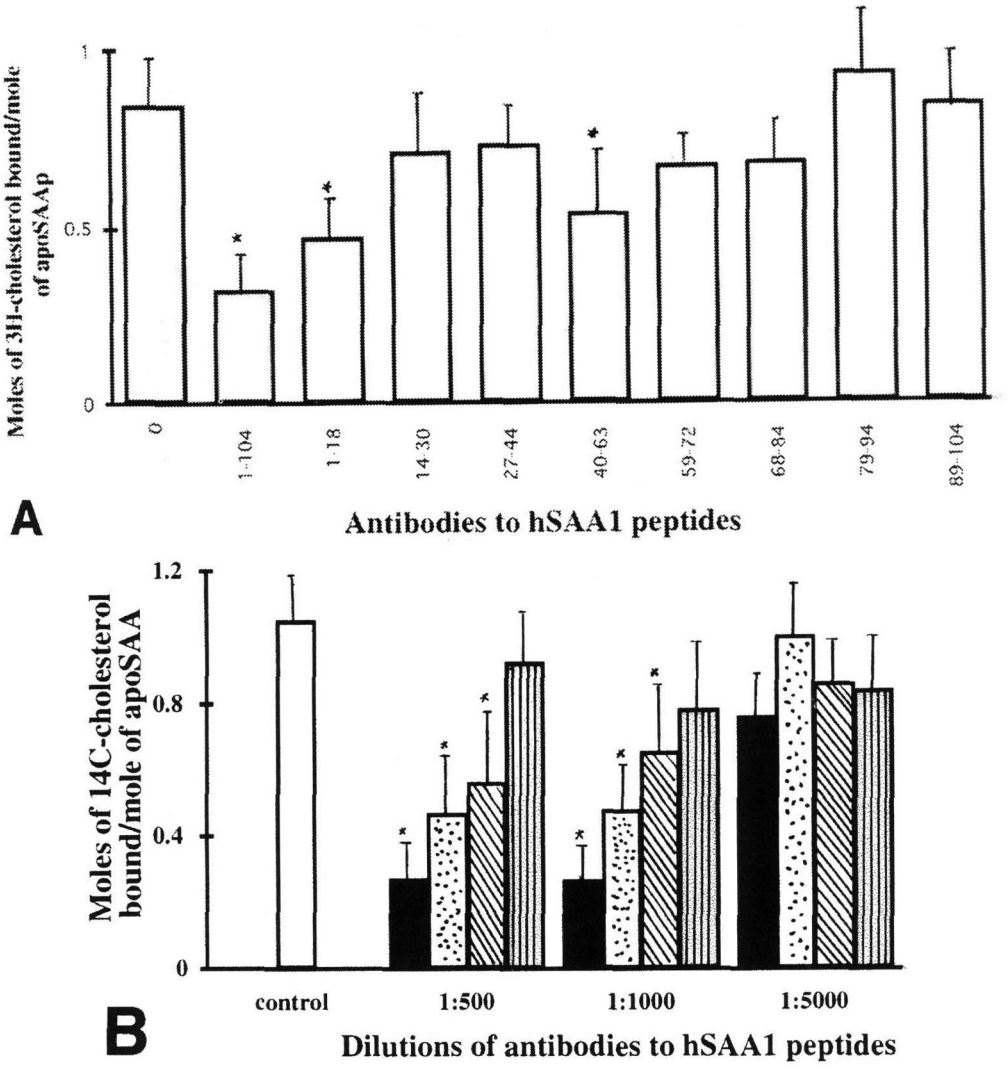

Fig. 4. A: Effect of antibodies to various regions of apoSAA on [^{3}H]cholesterol binding to apoSAA$_p$. To measure the effect of antibodies to apoSAA on cholesterol binding to apoSAA$_p$, apoSAA$_p$ (167 pmol) was incubated at 37°C in 1 ml serum-free RPMI with rabbit polyvalent antibodies raised to one of a group of eight synthetic peptides corresponding to overlapping regions of amino acids 1–18, 14–30, 27–44, 40–63, 59–72, 68–84 or 89–104 of apoSAA$_1$ (1:1000 dilution). Two hundred fifty pmol of [^{3}H]cholesterol was then added and the samples were incubated at 25°C for 18 h. The apoSAA$_p$–cholesterol complexes were isolated and measured as described in Materials and Methods. The data are expressed as mean ± standard deviation (SD). This experiment was performed three times. The data were analyzed by one-factor analysis of variance. *$P < 0.05$, compared with control (without antibodies). B: Dose-dependent effect of antibodies to specific segments of apoSAA on [^{14}C]cholesterol binding to apoSAA$_p$. The cholesterol binding assay was performed as described in Materials and Methods. Control (□); AB1-104 (■); AB1-18 (▨); AB40-63 (▧); AB89-94 (▦); Comparison of the data by two-factor analysis of variance demonstrate that both antibody and dose significantly alter cholesterol binding by apoSAA. Data are expressed as mean ± SD. This experiment was performed three times. *$P < 0.05$, compared with the control.

indicate that part of the cholesterol taken up by SMC in the presence of apoSAA is esterified. Moreover, cholesterol uptake by human neuroblastoma cells and human melanocytes was not enhanced in the presence of either apoSAA or a synthetic peptide corresponding to residues 1–18 of human SAA$_1$. These two findings suggest cellular specificity of apoSAA enhanced cholesterol uptake by SMC and HepG2 cells that may involve apoSAA specific binding sites.

SMC are active participants in the pathology of atherosclerosis. Our finding that the amino terminal region of acute phase apoSAA specifically binds and transports cholesterol into SMC may have pathophysiologic significance in view of previous reports of the

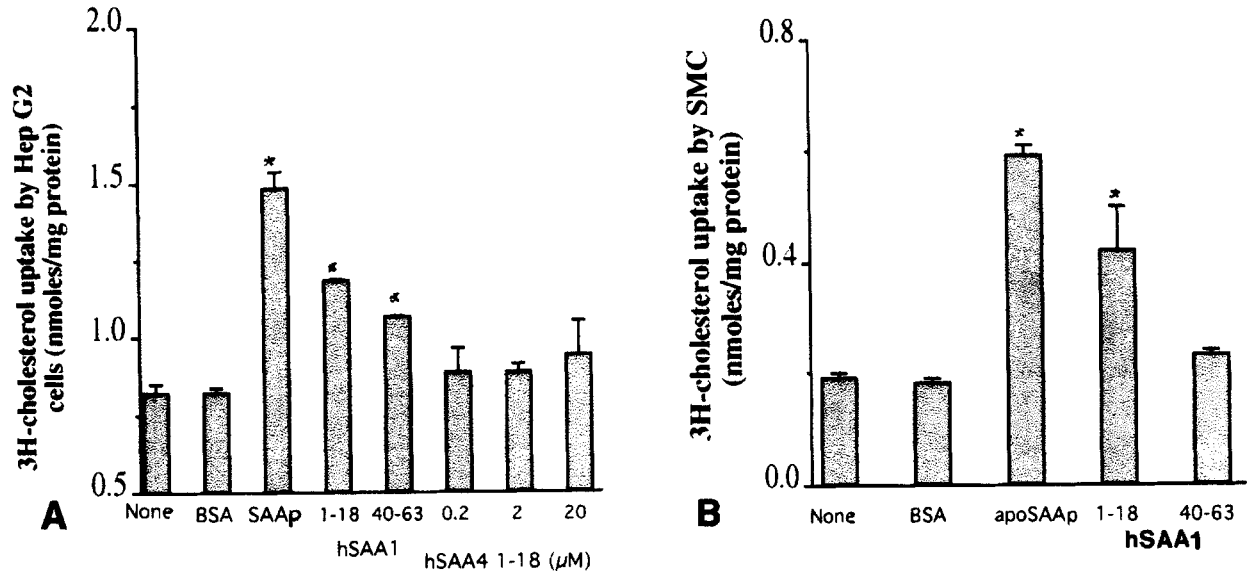

Fig. 5. A: Enhancement of [^{3}H]cholesterol uptake in HepG2 cells by apoSAA$_p$ and peptide corresponding to residues 1–18 of hSAA$_1$. HepG2 cells were washed thrice with serum-free DMEM medium and then incubated with 0.4 ml medium containing [^{3}H]cholesterol in the presence or absence of synthetic peptides (hSAA$_1$ 1–18 and 40–63, 2 μM; hSAA$_4$ 1–18, 0.2–20 μM), apoSAA$_p$ (2 μM) or BSA (40 μg/ml, 0.6 μM) in triplicate wells for 24 h. Culture medium was removed, each well was washed thrice with serum-free DMEM, 0.5 ml of Gel and Tissue Solubilizer was added, followed by incubation at 70°C for 4 h. Triplicate 50-μl aliquots were removed for counting of radioactivity and triplicate 10-μl aliquots for protein assay. Data are expressed as pmol/mg cell protein ± SD. *$P < 0.05$, compared with the control (none). This experiment was performed three times. Each well of HepG2 cells contained 264 ± 26 μg cell associated protein. No significant difference in cell associated protein concentration between control and experimental wells was observed after the 24 h incubation. B: Enhancement of [^{3}H]cholesterol uptake in SMC by apoSAA$_p$ and a synthetic peptide corresponding to residues 1–18 of hSAA$_1$. SMC were washed thrice with serum-free DMEM media and then incubated with 0.4 ml media containing [^{3}H]cholesterol in the presence or absence of synthetic peptides (2 μM), apoSAA$_p$ (2 μM) or BSA (40 μg/ml, 0.6 μM), in triplicate wells for 24 h. Culture medium was removed, each well was washed thrice with serum-free DMEM, 0.5 ml of Gel and Tissue Solubilizer was added, followed by incubation at 70°C for 4 h. Triplicate 50-μl aliquots were removed for counting of radioactivity and triplicate 10-μl aliquots for protein assay. Data are expressed as pmol/mg cell protein ± SD, *$P < 0.05$, compared with the control (none). This experiment was performed twice. Each well of SMC contained 196 ± 21 μg cell associated protein. No significant difference in cell associated protein concentration between control and experimental wells was observed after the 24 h incubation.

presence of apoSAA in atherosclerotic lesions (3). Although virtually all apoSAA in the circulation is associated with HDL (14), there is evidence from a number of in vivo and in vitro studies that apoSAA may dissociate from HDL in peripheral tissues prior to catabolism (15–17). In addition to its rigid ring system, cholesterol contains an –OH group at C-3 and an aliphatic side chain at C-17. These features make it more rigid than other membrane lipids. It has been suggested that the subcellular distribution of cholesterol in membranes is regulated by membrane proteins (18). It is possible that apoSAA proteins interact with cholesterol in the plasma membrane or alter the extracellular cholesterol concentration and hence the diffusional gradient of free cholesterol, thereby contributing to cholesterol uptake by the cells.

ApoSAA contains amphipathic helical regions. Segrest and coworkers (19) described the amphipathic properties of the first 26 amino acids of a 45 amino acid fragment of human apoSAA, an observation that led to the discovery of apoSAA as an apolipoprotein. Later, the first 11 of the amino terminal amino acids were predicted by secondary structure predictive analysis to be involved in lipid binding (11, 12). Synthetic peptides corresponding to amino acids 1–11 of apoSAA$_1$ and apoSAA$_2$ were shown to be capable of forming amyloid fibrils under acidic condition in vitro. The diminished α helical structure in the amino-terminal region spanning residues 1–11 of acute phase apoSAA$_1$ and apoSAA$_2$ relative to apoSAA$_4$ (Fig. 2) suggests that the amount of helical structure may influence both binding to polystyrene surfaces and to cholesterol. The 40–63 region appears to play a lesser role in cholesterol binding by apoSAA. The secondary structure prediction of this 24 amino acid residue segment is 42% helical and 38% extended, whereas the region from residues 1–18 is 61% helical and 33% extended. This supports the concept that the aliphatic side chain of cholesterol may be interacting with helical regions, but it is unclear how apoSAA forms a dimer upon binding a molecule of cholesterol (7).

Both SMC and HepG2 cells would constitutively synthesize and secrete apolipoproteins, but not apoSAA in the absence of cytokines. The uptake of cholesterol in

the absence of apoSAA (control) may be influenced by these apolipoproteins; however, this basal uptake of cholesterol was not addressed by the present study. As HDL are believed to be the physiological acceptors of cholesterol, it is to be expected that any disturbance in HDL composition may compromise cholesterol homeostasis and ultimately increase the risk of atherosclerosis. During the acute phase response, apoSAA_1 and apoSAA_2 can transiently constitute as much as 80% of the total HDL proteins and displace the primary protein constituent of HDL, apoA-I (20). That fact, together with our present finding that there is specific binding of cholesterol by acute phase apoSAA_p, suggests that apoSAA may temporarily alter cholesterol transport between cells and plasma during the acute phase response. Basic studies of the interaction of constitutive and acute phase apoSAA isoforms with cholesterol and HDL will advance our understanding of how clearance of cholesterol from the artery wall is influenced when apoSAA concentrations are elevated. The presence of acute phase apoSAA on HDL is of pathophysiologic significance when either extremely high levels are produced, such as following myocardial infarction, or when lower levels are produced for prolonged periods of time such as in rheumatoid arthritis where cardiopulmonary problems are known to be a cause of early death.

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